Determinants of achieved LDL-cholesterol and 'Non-HDL' cholesterol in the management of dyslipidaemias

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Abstract

Purpose of the review – The advent of combination therapy to provide LDL lowering beyond that achieved with statins necessitates the development of greater understanding of how drugs work together, what changes occur in key lipoprotein fractions, and what residual risk remains.

Recent findings – Clinical trials of agents that, when added to statins, generate profound LDL lowering have been successful in reducing further the risk of cardiovascular disease. LDL cholesterol can be now decreased to unprecedented levels, so the focus of attention then shifts to other apolipoprotein B–containing, atherogenic lipoprotein classes such as Lipoprotein (a) and remnants of the metabolism of triglyceride-rich particles. 'Non-HDL cholesterol' is used increasingly (especially if measured in the non-fasting state) as a more comprehensive index of risk.

Summary – Metabolic studies reveal how current drugs act in combination to achieve profound lipid lowering. However, care is needed in interpreting achieved LDLc and Non-HDLc levels in the emerging treatment paradigm.

Introduction

Cholesterol in the bloodstream is transported in lipoproteins that can be divided broadly according to structure and function into apoB-containing particles and apoA-containing particles. The former lipoprotein class which includes LDL -the most abundant cholesterol transporter in healthy individuals - is linked to increased risk of atherosclerosis and CHD (1-4*) and there is abundant evidence to support the causal nature of this relationship (3**). The latter – HDL - present an enigma; this fraction exhibits many cardio-protective features, is strongly, inversely associated with risk of CHD (5*), but trials in which it is increased through pharmacological modulation have not shown clinical benefit (5,6). Current management strategies for prevention of cardiovascular disease, therefore, focus primarily on reducing levels of apoB-containing lipoproteins in the circulation (1,2).

Evidence from epidemiology, pathology, genetics, and clinical outcome trials provides a coherent picture of LDL as a, if not 'the', causative factor in atherogenesis. The association of LDL cholesterol (LDLc) with CHD incidence is continuous, log-linear and appears to have no 'floor' i.e. a lower limit below which further reduction does not lead to decreased risk of CHD (3, 7). This is in distinction to blood pressure, a comparable, prevalent risk factor in the population that needs to be maintained within an optimal, physiological range. The HOPE-3 trial demonstrated clearly that LDLc lowering but not blood pressure lowering resulted in risk reduction even when subjects had average levels of these factors (8). Further support for this concept came from the FOURIER study which showed that addition of a proprotein convertase: subtilisin kexin 9 (PCSK9) inhibitor (evolocumab) to statin therapy to reduce LDLc to very low levels led to a proportionate decrement in CHD risk (9**,10**).

LDL is the end product of a metabolic cascade in which triglyceride-rich very low density lipoprotein (VLDL) is progressively delipidated through the action of lipases. In some people

this is extremely efficient, whereas in others – those with elevated plasma triglyceride levels - delipidation is slower and the prolonged residence time of VLDL particles allows them to accumulate cholesteryl ester by transfer from other lipoproteins via the agency of cholesteryl ester transfer protein (CETP), and 'remnant' lipoprotein species are formed (11,12). In a similar fashion, lipolysis of chylomicrons released from the intestine in response to a diet fat load leads to the formation of cholesterol-rich, more slowly metabolized remnants. Particles from both sources are believed to be atherogenic since they contain apoB (VLDL remnants have apoB100, and chylomicron remnants apoB48, as the major structural protein), a cholesterol 'payload', and can permeate the arterial wall sub-endothelial space where lesion formation is initiated (11,12). There is increasing evidence that the plasma concentration of remnants is related to CHD risk independent of LDLc level (4, 11-14).

Lipoprotein (a) (Lp(a)) is a separate apoB100-containing lipoprotein class with a metabolic origin and fate distinct from that of the VLDL-LDL delipidation pathway (15). Genetic studies have reinforced the conviction that Lp(a) is a causal risk factor for CHD and therefore a worthwhile therapeutic target (16,17*). There has been controversy over the quantitative nature of the association of plasma Lp(a) with risk but recent comprehensive evaluations indicate that a rise of 100mg/dl in its concentration (measured as total lipoprotein mass) gives a risk approximately equivalent to an increase of 1.0 mmol/l (39mg/dl) in LDLc (17,18).

The LDLc fraction as routinely assayed in the laboratory (see below for a more detailed discussion on methodology) incorporates LDL particles of varying size and composition, intermediate density lipoprotein (IDL), and Lp(a) (Figure 1). Non-HDL cholesterol (Non-HDLc) calculated as the difference between total and HDL cholesterol is increasingly favoured as a measure of the entire atherogenic potential of plasma lipoproteins. It includes

in addition to the LDL fraction, the cholesterol present in VLDL/remnant lipoproteins. Since, as noted above, all of these apoB containing species can contribute potentially to formation of atherosclerotic lesions, they are suitable targets for therapeutic invention with diet and drugs, and it may be that until all these particles are reduced to acceptable (minimal?) levels then lipoprotein-associated CHD risk will not be adequately controlled.

Action of lipid lowering drugs on LDL- and Non-HDL cholesterol.

Kinetic studies such as those depicted in Figure 1 have been undertaken in an effort to understand the regulatory mechanisms that control the abundance of VLDL, VLDL remnants, IDL and LDL in the bloodstream, and the actions of lipid-modifying drugs (19, 20). Achieved LDLc and Non-HDLc levels are determined by individual responses to agents administered as monotherapy or in combination. Evidence from studies so far supports the general concept that the effects of commonly used drugs on lipoprotein metabolism are additive (the net effect is the sum of individual actions) in distinction to being synergistic, or offsetting (that is, addition of a second agent interferes with or cancels the action of the first).

Metabolic investigations have revealed that statins, ezetimibe and PCSK9 inhibitors share a common mechanism for lowering the levels of LDL and other apoB-containing lipoproteins, namely increased activity of the LDL receptor. It has been known for many years that inhibition of cholesterol synthesis with statins, particularly in the liver, leads to a fall in regulatory cholesterol pools and increased transcription of LDL receptors. This in turn promotes uptake of LDL particles by cells to replenish the depleted intracellular cholesterol (Figure 1). Ezetimibe has its

primary effect in the intestine where it blocks uptake of luminal cholesterol by enterocytes (21). It is believed that this leads to decreased transport of the sterol to liver in the chylomicron pathway and consequently a fall in hepatic cholesterol and a rise in receptor activity. Up-regulation of LDL clearance appears to be the main mechanism by which ezetimibe reduces LDLc (22).

The LDL receptor is not the only gene product influenced by pharmacologically induced changes in intracellular cholesterol; expression of the gene coding for PCSK9 is also increased during statin administration (23). This protein appears to have both intra- and extra-cellular functions related to lipoprotein metabolism (23,24). The major action of circulating PCSK9 is to bind to LDL receptors and promote degradation, thereby decreasing their abundance on the cell surface and causing a reduction in LDL particle clearance rate (23-26). This pathway is quantitatively important; it can be calculated that in normal subjects about half of potential LDL receptor activity is blunted by the action of PCSK9 (25-27). The existence of a counter-regulatory mechanism whereby the statin-induced rise in PCSK9 partially offsets the stimulation of LDL receptor activity provides a satisfactory explanation as to why LDLc is lowered only modestly (by about a further 6%) as the dose of statin is increased (doubled) (28).

As shown in Figure 1 (based on investigations reported in 25**,26**), statin treatment in normolipemic volunteers caused substantial falls in IDL and LDL apoB pool sizes, and a modest decrement in VLDL apoB, due to increased clearance of these lipoprotein classes. A qualitatively and quantitatively similar effect was observed when a PCSK9 inhibitor was given as monotherapy (25,26). Subjects

treated with statin plus PCSK9 inhibitor demonstrated a profound reduction in LDL apoB pool size (Figure 1) - to 15% of the off-treatment value– due mainly to an additive effect of the two drugs on the LDL fractional clearance rate (25), while there was a significant but less dramatic impact of combination therapy on VLDL and IDL clearance rates. It was noteworthy also that IDL to LDL conversion was decreased by PCSK9 inhibition but not by statin therapy and this contributed to the fall in LDL apoB in both the mono- and combination- therapy settings (25,26).

Recent clinical trials of combination therapy - statin plus PCSK9 inhibitor (plus ezetimibe in some cases) - reveal that, as in the healthy subjects in Figure 1, LDLc in patients at very high risk can be reduced routinely to extremely low levels (29-31). Further, those with heterozygous familial hypercholesterolemia (FH) in whom LDL receptor expression is compromised can achieve normal or even low-normal LDL levels as the addition of a PSCK9 inhibitor to statin stimulates maximal expression of the remaining functional allele (32). There is, however, emerging evidence that statins and PCSK9 inhibitors may have differential effects in certain hyperlipidemic states. It is known that in patients with elevated plasma triglyceride levels, statins lower VLDL, IDL and LDL to approximately the same degree (20,33); the percent reduction in plasma triglyceride in those with levels of this lipid in the 1.5-4.0 mmol/l range is about the same as the percent decrease in LDLc (33). This is attributable to a statin mediated increase in the clearance rate of VLDL that matches that of IDL and LDL particles (20) (in distinction to what is seen in normotriglyceridemics (Figure 1, 20)). Preliminary analysis of PCSK9 inhibitor trials indicates that the decrease in plasma triglyceride (VLDL) is less marked than that of LDL in hypertriglyceridemic patients (34). This may point to a preferential action of

these agents on the clearance pathways of smaller (LDL sized) particles, or an additional effect of statins on VLDL catabolism. In terms of determining achieved lipid levels, these observations suggest that in hypertriglyceridemic subjects e.g. those with obesity or type 2 diabetes, PCSK9 inhibitors may not correct fully the atherogenic dyslipidemic profile even when added to statin therapy. The concept that a residual risk may be attributed to lipoprotein species in the VLDL density range even when achieved LDLc is very low finds support in observations from the JUPITER trial - when LDLc was reduced to a mean of 54mg/dl with rosuvastatin, the concentration of small VLDL was linked to ongoing risk (35*) – and in an evaluation of the effect of evolocumab on lipoprotein particles. Toth et al (36) reported an overall decrease in VLDL/ chylomicron particle number of only 15% compared to a fall of 44% in LDL. It was noted also that there was a greater effect of the PCSK9 inhibitor on the larger LDL, and smaller VLDL, sub-species (36).

Lipoprotein (a) comprises an LDL particle to which is covalently bound apolipoprotein (a) (apo(a)), a highly variable peptide made up of differing numbers of 'kingle' subunits. Apo(a) appears to interfere with the affinity of apoB for the LDL receptor and thereby alters the clearance route of the particle (15,16). The observation that statins failed to decrease circulating Lp(a) levels added to the conviction that the LDL receptor was not involved in its catabolism (16). However, the finding in clinical trials that PCSK9 inhibitors can reduce Lp(a) by about 25-30% has led to a re-evaluation of this concept (29,30,37**). There is evidence from cell culture studies that in appropriate conditions Lp(a) has access to the LDL receptor pathway and its uptake can be blocked by PCSK9 (37). Also, some human kinetic studies have revealed a trend to increased Lp(a) clearance on PCSK9 inhibitor

therapy (26). So, a current hypothesis is that Lp(a) has a low but not absent affinity for the LDL receptor and this clearance pathway becomes active when LDL particle numbers are profoundly reduced (Figure 1). PCSK9 inhibition is therefore an appropriate therapeutic strategy in high-risk subjects with elevated Lp(a) levels on maximal statin treatment (37).

CETP inhibitors are drugs that not only raise HDLc by about two-fold but also lower LDLc and Non-HDLc (6,38**). The significant effect on apoB-containing lipoproteins was unexpected since variation in the gene for CETP was initially found to be associated primarily with change in HDL but not in LDLc or plasma triglyceride (39). However, a more comprehensive genomic analysis revealed that some allelic variants were linked strongly to reductions in LDLc (40**). CETP mediates the bi-directional exchange of cholesteryl ester and triglyceride between lipoproteins and its usual action is the net transfer of cholesteryl ester from HDL to chylomicrons, VLDL and LDL. Hence, when it is blocked almost completely with drugs like anacetrapib, HDLc rises dramatically and there is a corresponding decrease in the cholesterol content of LDL. It follows that a component of the decrement in achieved LDLc with CETP inhibitors is due to compositional change in the lipoprotein. However, since plasma apoB falls about 15 to 20% there must also be a decrease in number of particles (6,38,40,41). Kinetic studies have attributed the latter to an effect on LDL clearance which shows about a 20% increase on anacetrapib (42). Since CETP inhibition appears to impact LDL particle abundance in the same manner as the other drug classes described above, it is tempting to speculate that it does so by reducing cholesterol in a key hepatic regulatory pool.

Mendelian randomization studies of genetic variants that mimic the actions of statins, ezetimibe, and PCSK9 inhibitors have been used to demonstrate that regardless of the means used to lower LDLc there is a consistency in the impact on CHD risk (3); when inherited variation is standardized to a fixed LDLc change e.g. 10mg/dl, the decrease in risk at 18% is the same for all 3 genetic loci (43). This observation extends to CETP inhibition when apoB is used as the measure of lipid lowering, change in LDLc at least in those on background statin therapy appears to overestimate the risk reduction (40). These seminal genetic observations complement the meta-regression analysis that shows that across all lipid lowering trials with statins, ezetimibe and PCSK9 inhibitors the decrease in risk per unit change in LDLc falls on the same regression line (3,10,43,44). This information is highly useful in understanding what has been achieved for a given degree of LDLc reduction, and what modifiable residual risk remains.

Composition and measurement of LDL and non-HDL cholesterol on lipid-lowering therapy.

When receiving a laboratory report of achieved lipid levels, the clinician is faced with the questions - 'Is this the expected response to the prescribed therapy?" and "What more needs to be done given the hyperlipidemic- and risk- status of the patient?" It is important therefore to interpret the result in light of how the prescribed drugs act, the magnitude of the expected change, and any known shortcomings in the assay procedure. This is especially the case if the patient is on optimized first-line treatment – usually a statin - and not at goal, and a decision has to be made as to whether to proceed to combination or alternate therapy (45,46). Further, with the unprecedented ability to drop LDLc to very low levels there needs to be greater appreciation of the limitations of current assay techniques. Laboratory tests

designed to provide reasonably accurate results in the usual range of values seen in the population might not function well when profound lipid lowering is undertaken. There are a number of approaches to assessing the concentration of apo-B containing lipoproteins in plasma, the most common by far is to measure their cholesterol content, the second, arguably more informative, method is to assay apoB itself, and the third is to employ novel technologies to determine particle number and size distribution. Accordingly, there are a number of factors, described below, that clinicians now need to take into account in interpreting results.

Fasting versus non-fasting LDLc estimation

Classically, lipids have been measured after an overnight fast and the resultant profiles used to characterize dyslipidemias and predict risk of CHD. However, recent investigations have explored whether fasting is essential to classify an individual's risk status, and indeed the extent to which a non-fasting test provides a superior index of risk (4,11-14). Recently, the European Atherosclerosis Society and the European Federation of Clinical Chemistry and Laboratory Medicine concluded in a joint statement that the use of non-fasting samples provides an assessment of risk that is at least as robust as that gained from fasting analysis (47*). The report highlighted the fact that directly measured LDLc differs little between fasted versus non-fasted state, and its estimation from the overall lipid profile (plasma cholesterol, triglyceride, HDLc) using approaches such as the Friedewald equation provide satisfactory results for the general population (47); calculated LDL from non-fasting subjects showed a mean difference of -0.2 mmol/l compared to measured levels. However, other investigators addressing this topic have demonstrated systematic bias in Friedewald derived LDLc in the non-fasting state (48**), and when LDLc is <1.8 mmol/l (<70mg/dl) (48,49)). A novel calculation has been proposed that adjusts the factor used for the VLDL cholesterol: triglyceride ratio (1:5 in the Friedewald calculation) according to the levels of

Non-HDLc and plasma triglyceride (48). This stratified approach improved the accuracy of achieved LDLc estimation, and hence prediction of a patient's residual risk.

Non-HDL- and remnant- cholesterol estimation.

Non-HDLc is considered a co-primary target (with LDLc) for lipid-lowering therapy by the National Lipid Association of the USA (50), is the primary focus for intervention in UK guidelines (51), and is an important subsidiary target in the joint European guidelines (2). This lipid fraction as noted above incorporates all apoB-containing lipoprotein species and is a convenient alternative to total plasma apoB measurement.

There is no universal agreement of the definition of remnant cholesterol. Some investigators view this fraction as 'total measured cholesterol minus measured HDLc minus estimated LDLc' i.e. all the cholesterol present in the VLDL (plus chylomicron) density ranges (4,11). An alternative conceptual definition is that remnant cholesterol is present in in a subfraction of partially-lipolysed triglyceride-rich lipoproteins that have a relatively long residence time (52). It is difficult to assess the cholesterol component of the latter since the metabolic characteristics of the particles do not translate easily into structural variations that would allow their separate measurement. Possibly, the closest corollary is the assay for remnant-like particle (RLP) cholesterol that is based on variation in apoB conformation and apolipoprotein content (4). Varbo et al explored whether VLDL cholesterol (Non-HDLc minus LDLc) or RLPc gave the better index of risk and found that while both were related to CHD in the Copenhagen Heart Study, the former exhibited the stronger relationship (4, 53).

In considering achieved lipid levels and residual risk, for example in patients on statins plus PCSK9 inhibitors, it is worthwhile noting changes in the relative contribution of LDLc and remnant cholesterol (in this case VLDLc) to the measured Non-HDLc. In Figure 2, based on findings reported for the ODYSSEY (29) and OSLER (30) studies, it can be seen that on

PCSK9 inhibitors remnant cholesterol accounts for a much greater proportion of the achieved Non-HDLc due to the greater impact of these agents on LDL versus VLDL metabolism (Figure 1). This will be especially true in subjects with elevated plasma triglyceride (VLDL) levels such as diabetics and those with combined hyperlipidemia. Arguably, therefore, as suggested by expert bodies Non-HDLc should receive as much attention as LDLc in devising treatment strategies.

LDLc and high lipoprotein(a)

It has long been recognized that the cholesterol contained in Lp(a) particles (about 30% of the total measured lipoprotein mass) contributes to the observed LDLc level whether the latter is estimated (calculated) from the overall lipid profile, or measured directly by ultracentrifugation (16). Traditionally, this issue was considered clinically relevant only when Lp(a) was high e.g. >50mg/dl total mass. However, with the ability to drive LDL down to very low levels using statin plus PCSK9 inhibitors and the differential effect of these drugs on LDL and Lp(a) particles (37), there is now a practical problem in that Lp(a) –associated cholesterol could be a major component of the measured LDLc, especially when levels are below 70mg/dl (54). Statins lower LDLc typically by 25 to 55%, and have little impact on Lp(a), or cause a slight increase (16). Ezetimibe lowers LDLc by 20-25% with a modest effect on Lp(a) (55) but PCSK9 inhibitors on average reduce LDLc by 60% and Lp(a) by 29%. Thus, in the scenarios explored by Yeang et al (54), patients with elevated Lp(a), say 90mg/dl (27 mg/dl cholesterol) given a PCSK9 inhibitor may exhibit an achieved LDLc of 35 mg/dl of which only 16mg/dl (i.e. after subtracting 19mg/dl for on-treatment Lp(a)) is 'true' LDL. Knowledge of Lp(a) will in these circumstances greatly influence interpretation of the achieved level. Having said that, there is probably no cause for concern that LDLc has been lowered so profoundly since recent investigations exploring potential adverse effects of very low LDLc levels have not shown any safety signals even when <25mg/dl, and indeed continuing reductions in risk to <10mg/dl (31,56*,57*).

Conclusion - Assessing achieved LDLc and Non-HDLc and clinical implications.

With the advent of PCSK9 inhibitors there is now an unprecedented ability to treat patients with hypercholesterolemia, especially the inherited condition of familial hypercholesterolemia (FH). Clinical trials indicate that LDLc levels in these patients can be reduced to goals recommended for the general population and those at high risk with established CHD (32,58). The magnitude of the reduction in LDLc appears independent of genetic status, and even those with homozygous FH respond as long as they have LDL receptors with some functionality (32,59). Since hypercholesterolemic patients will likely have elevated levels of VLDL remnants and IDL, and in many cases increased Lp(a) (32), it is important to perform a full lipoprotein profile when achieved LDLc is low, <70mg/dl (<1.8 mmol/l) or Non-HDLc is <100mg/dl, including direct measurement of LDLc, assay of apoB, and Lp(a).

Subjects with hypertriglyceridemia will generally respond well to statins (33) with a reduction across all apoB-containing lipoproteins (20), and will improve further if given ezetimibe and/or PCSK9 inhibitors (29,30). These subjects will have also a preponderance of remnants and small, dense LDL in the LDL density range if the plasma triglyceride concentration remains above normal (>1.5mmol/l) and on that basis, may have continuing high risk of CHD.

At present, there is limited evidence that common genetic variants, or subjects' characteristics such as body weight or age have enough of an impact on the response to statins, ezetimibe or PCSK9 inhibitors to influence treatment decisions (1,2). However, it should be noted that while the average percent LDLc decrease for a given statin dose and potency is reasonably predictable (60) individual responses can vary markedly. One of the largest studies to examine this is the JUPITER trial where 'waterfall' plots showed LDLc

change on a fixed dose of 20mg rosuvastatin ranged from a modest increase/no change to >80% reduction (61). Similar wide variation was seen in percent change in Non-HDLc and apoB. In JUPITER predictors of lower on-treatment LDLc included male sex, Caucasian ancestry (61,62), and higher age and body mass index (61). In an exploration of the genetic determinants associated with variation in LDLc response to rosuvastatin, 4 loci were identified as significant – ABCG2, LPA, APOE and PSCK9 (63), and ranking subjects in groups according to the number of alleles linked to lower (baseline) LDLc gave a range of mean percent reduction from about 44% to >58%. So, individual gene effects while small in magnitude may be important in aggregate in determining not only the LDLc change but also clinical benefit. While it is observed in global meta-analysis that the risk reduction per unit change in LDLc is relatively consistent regardless of patient type and starting LDLc (64), recent studies indicate that subjects with a high cardiovascular gene score (comprised of variants linked to increased CHD incidence) experience a greater relative risk reduction for a given change in LDLc (65,66). As this information is refined and confirmed, genetic testing may indeed in the future form part of the treatment algorithm especially in primary prevention.

In summary, the emerging treatment paradigm of profound cholesterol lowering with combination therapy affords the clinician a wider range of options when dealing with difficult to treat or very high risk patients. Appropriate assessment of what has been achieved in terms of decrements in LDLc, Non-HDLc, remnant lipoprotein levels and Lp(a) is needed in order proceed with confidence. We still require a more complete picture of the effect of PCSK9 inhibitors on the metabolism of VLDL and chylomicrons in order to appreciate the full potential of these agents in hypertriglyceridemic states, and the unmet need that remains to be addressed by agents still in development (67).

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Figure legends

Figure 1. Flowchart of apolipoprotein B metabolism in normolipemic subjects on no therapy, statin or PCSK9 inhibitor monotherapy and combination therapy (statin +PCSK9 inhibitor)

The schematic depicts VLDL, IDL and LDL particles arranged in a delipidation cascade. The VLDL class includes a remnant lipoprotein species ('R'). Lipoprotein (a) is shown as a separate entity; it is derived from the liver but has distinct metabolism in the untreated state (No Rx). LDLc is shown as including IDL, LDL and Lp(a). Non-HDLc includes all of the particles in LDLc plus cholesterol in VLDL + remnants.

The illustrative changes in rates/ pool sizes (e.g. 1.2x; 50% decrease) on statin, PCSK9 inhibitor or on a combination of the two drugs are relative to the basal state (derived from references 25, 26). VLDL, IDL and LDL particles are removed from the circulation via the action of cell surface receptors, principally the LDL receptor. Statin or PCSK9 inhibitor alone increased the clearance rates of LDL, IDL and to some extent VLDL. Combination therapy accentuated this action, particularly promoting LDL clearance (25). PCSK9 inhibition decreased the conversion of IDL to LDL (25,26). Lp(a) is depicted as being reduced by PCSK9 inhibition through stimulated clearance by receptors (26,37).

Figure 2. Response of LDLc and Non-HDLc to PCSK9 inhibitor therapy.

Change in Non-HDLc, LDLc, plasma triglyceride and remnant cholesterol levels in the ODYSSEY Long Term (29) and OSLER (30) studies. 'Remnant cholesterol' is this instance was the total calculated cholesterol in the VLDL fraction (total plasma cholesterol – HDLc – LDLc).

Compliance with Ethics guidelines

There are no ethics issues relevant to this review article.

Conflict of interest

The author has received grants/ honoraria from the following pharmaceutical companies – Merck, Sharp & Dohme, Pfizer, Amgen, Sanofi, Regeneron, Daiichi-Sankyo.

Human and animal rights and informed consent

This article does not report any studies with human or animal subjects performed by the author.